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EXOGENOUS NUCLEOTIDE PROTECTION OF MAMMALIAN CELLS FROM DIPHTHE--ETC(U)
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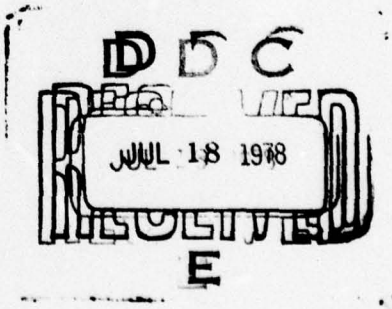

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ABSTRACT

Exogenous nucleotides were found to protect mammalian cells from the lethal effects of diphtheria toxin. Protective potency of a given nucleotide was base-specific and phosphate chain-length dependent. Full expression of protective potency required an intact nucleotide, but the effect did not appear to be mediated by nucleotide-induced phosphorylation. Nucleotides antagonized the binding of diphtheria toxin to its cell surface receptor in a manner that correlated with the degree of protection. It was concluded that cellular protection from diphtheria toxin by nucleotides results from inhibition of toxin-receptor binding and that nucleotides therefore may serve as valuable research tools for future studies.

KEY WORDS Diphtheria toxin. receptor. mammalian cells. nucleotides.

Normally, the eukaryotic cell membrane acts as an efficient barrier to the intracellular passage of intact macromolecules. However, it is clear that several toxins and probably some polypeptide hormones cross the membrane in order to carry out their biological actions. Diphtheria toxin (1), Pseudomonas aeruginosa exotoxin A (9), abrin (16) and ricin (16) must in some manner reach the cytosol to express their toxicity. There is a possibility that insulin (22) and various mammalian cell growth factors (8) also act intracellularly. Thus, insights into the mechanism by which any one of these proteins interact with and cross the cell membrane should have widespread interest in cell biology.

As previously pointed out (1), diphtheria toxin is well suited to a study of the mechansim by which a biologically active macromolecule crosses the cell membrane. This toxin is lethal for several animal species and cytotoxic for a variety of cultured mammalian cells (4, 7, 17). Toxicity is expressed via inhibition of eukaryotic cellular protein synthesis (20), resulting from inactivation of a cytoplasmic enzyme required for protein synthesis, elongation factor-2 (EF-2) (4). A 21,000 dalton fragment (fragment A) of diphtheria toxin (62,000 daltons) is a transferase which covalently links the ADP-ribose portion of NAD^+ to EF-2; ADP-ribosylated EF-2 is inactive at protein synthesis. The remaining portion of diphtheria toxin (fragment B) has no reported biological activity, but is apparently required for the initial interaction of the toxin with its target cell membrane (4, 7, 17). Thus it is generally believed that diphtheria toxin binds to a cellular receptor by fragment B followed by traversal of the cell membrane and expression of enzymatic activity by fragment A.

Until recently, little was known about the binding of diphtheria toxin to its receptor or the transport of the toxin (toxin-receptor complex?) across the cell membrane. Boquet and Pappenheimer (1) studied the interaction of radiolabeled diphtheria toxin with HeLa cells and found a specific uptake of the label. We recently found that Vero cells have large numbers of diphtheria toxin-binding sites and that binding of toxin to these sites exhibits biophysical and biological features consistent with a specific toxin-receptor interaction (13). We report here that exogenous nucleotides effectively protect cells from the action of diphtheria toxin, apparently by competetive antagonism of toxin-receptor binding. It is suggested that nucleotides can therefore serve as unique tools for future diphtheria toxin-receptor studies, and may be valuable in investigations of other toxin- or hormone-responsive systems.

MATERIALS AND METHODS

Cells and Cell Culture

Seed stock for all cell lines was obtained from the American Type Culture Collection (ATCC), Rockville, Md. Each line was maintained in 75-cm^2 T-flasks (Costar #3075) with the medium and serum supplement recommended by ATCC.

Media and Sera

All media, vitamins, antibiotics and amino acids were obtained from Grand Island Biological Company, Grand Island, N.Y. Fetal calf serum was purchased from Reheis Chemical Company, Phoenix, Ariz. The serum was heat-inactivated for 30 min at 56°C before use in cell culture.

Diphtheria toxin was obtained from Connaught Laboratories (Toronto) and purified by chromatography over DEAE-cellulose. The final product was indistinguishable in cell culture experiments from purified diphtheria toxin (23 MLD/μg) supplied by Dr. A. M. Pappenheimer, Jr., Harvard University. Toxin concentration was determined using an extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm of 11.9.

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) or PL Biochemicals (Milwaukee, Wis.). Concentrations were determined spectrophotometrically, using either published extinction coefficients (5) or those furnished by the supplier.

Cytotoxicity Assay

Details of our cytotoxicity assay have been described (14); a slight variation of the method was used for this work. Toxin and experimental chemical agents were added simultaneously to cells in 24-well (Costar #3524) tissue culture plates (triplicate samples) and

incubation was carried out at 37°C for 3 h. All nucleotide solutions were prepared in a 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer and adjusted to pH 7.2 $\pm$ 0.2 prior to addition to cells. The incubation was terminated by washing each monolayer three times with serum-free medium and adding complete medium to continue cell culture. After 48 h of further incubation, the monolayers were washed with Hanks' balanced salt solution and the remaining adherent cells were dissolved in 0.1 M NaOH for protein assay. Data are plotted as percent of control incubations with the chemical agent alone; standard errors were usually 1-4% (14).

Toxin-receptor Binding Assay

A detailed description of the binding assay has recently appeared (13). On the day of experimentation, the growth medium over cells in 24-well tissue culture plates was changed to 1 ml of Hanks' 199, supplemented with 10% fetal calf serum and 25 mM Hepes, pH 7.4. This medium change allowed direct comparison between experiments run at 4°C and 37°C in the absence of CO_2. Either ^{125}I-toxin or ^{125}I-toxin plus a 100-fold excess of unlabeled toxin was added to the wells (three replicates) and incubation was carried out under the conditions indicated. Each monolayer was then rinsed four times with Hanks' balanced salt solution, dissolved in 1.0 ml of 0.1 M NaOH and counted in a 1185 automatic gamma counting system (Searle Analytic Inc.). Standard errors were usually 1-3%. Specific binding is calculated as the difference between cell-associated counts in the presence or absence of unlabeled toxin.

RESULTS

Protection from Diphtheria Toxin by Adenine Nucleotides

Fig. 1A shows the dose-response relationship for exogenous adenine nucleotide protection of Vero cells from diphtheria toxin. Protection was dose-dependent and proportional to the length of the nucleotide phosphate chain. In the absence of nucleotide, diphtheria toxin killed about 75% of the cells. At low concentrations nucleotides had little or no effect on cytotoxicity, but at higher levels protection was virtually complete. As determined by the concentration required for 50% protection, adenosine-5'-tetraphosphate was the most potent protective agent of the series, about 5-8 times more potent then adenosine-5'-triphosphate (ATP). ATP was 2-3 times more potent then adenosine-5'-diphosphate (ADP), while adenosine-5'-monophosphate (AMP) exhibited incomplete but measurable protection. Neither the nucleoside, adenosine, nor the free base, adenine, at concentrations up to 1 mM, exhibited protective potential. The phosphate oligomer portion of ATP, tripolyphosphate (PPP), conferred protection at high concentrations.

The effects of adenine nucleotides on toxin-cell binding are shown in Fig. 1B. Clearly, nucleotide effects on binding can be correlated to effects on cytotoxicity. The relative potencies are the same, i.e., tetraphosphate > triphosphate > diphosphate > monophosphate. Furthermore, the concentrations of nucleotides required to protect 50% of the cells are close to those required to inhibit 50% of the binding, indicating similar absolute potencies as well. One anomaly, however, was the increase in binding brought about by adenine or adenosine. At 1 mM, these compounds increased the binding 20-30% over the control values (data not shown).

Specificity of the Nucleotide Base

Nucleotide-mediated protection from diphtheria toxin was not limited to adenine nucleotides. Fig. 2A shows that all the common purine and pyrimidine triphosphate ribonucleotides protected cells to some degree. In diphtheria-toxin challenged Vero cells, thymidine-5'-, adenosine-5'- and guanosine-5'-triphosphates were the most potent protective nucleotides. Cytidine-5'-triphosphate did have a protective effect but it was much less than the above three nucleotides. Uridine-5'-triphosphate and tripolyphosphate were about equally potent, falling midway between cytidine- and the other three triphosphates. Nucleotide-mediated inhibition of toxin-cell binding is shown in Fig. 2B. As with the adenine series, the correlation between effects on binding and cytotoxicity is readily apparent.

Protection from Diphtheria Toxin by Nucleotide Components

The presence of nucleotide components was not sufficient to achieve full protection, as demonstrated in Fig. 3A. Here, thymidine-5'-triphosphate (TTP) provided a high level of protection from a concentration of diphtheria toxin leading to substantial cell death. Neither thymidine nor tripolyphosphate provided protection, and together, their protective potential was not increased. Similar results were obtained with ATP and its components (data not shown). Thus, it appears that covalent attachment of tripolyphosphate to the nucleoside is necessary for full expression of toxin-protective activity.

A similar requirement is observed for inhibition of binding (Fig. 3B). Neither thymidine, tripolyphosphate, nor a combination of the two had appreciable effect on diphtheria toxin-cell binding. On the

other hand, a similar concentration of TTP blocked almost half of the toxin-cell binding exhibited by the control.

Nonhydrolyzable ATP Analogs

The correlation of nucleotide phosphate chain length to protective potency suggested that both protection from cytotoxicity and inhibition of toxin-cell binding may be mediated by phosphate transfer. To test this possibility, we investigated the effects of two nonhydrolyzable analogs of ATP; results are shown in Table I. In the presence of ATP, about 20% of the cells did not survive a concentration of diphtheria toxin that, by itself, kills about 80%. The ATP analog with a methylene linkage between the β-γ phosphates was slightly less potent than ATP while the analog with β-γ imido linkage was slightly more potent. All three compounds were effective and equipotent in their abilities to inhibit toxin-cell binding. It therefore seems highly unlikely that phosphate hydrolysis or transfer is responsible for either cell protection or inhibition of toxin-cell binding.

Competitive Nature of Binding Inhibition

To determine whether the nucleotide antagonism of toxin-cell binding was competitive or noncompetitive, binding was measured in the presence of two concentrations of ATP and a double-reciprocal analysis of the data was carried out. The results (Fig. 4), are consistent with competitive inhibition of diphtheria toxin-receptor binding. Competitive antagonism is usually interpreted as reversible binding of the antagonist (nucleotide) to the same site on a receptor as the agonist (diphtheria toxin) (23). However, allosteric effects cannot be ruled out.

DISCUSSION

Previous studies have shown that several drugs can protect mammalian cells from diphtheria toxin (6, 10, 15). This report identifies a class of naturally occurring compounds (nucleotides) as protective agents from diphtheria toxin and presents a limited structure-activity analysis of the chemical characteristics required for protection. One of the most striking features of nucleotide protection is the dependence on phosphate chain length. At least a 100-fold increase in protective potency is observed when going from a mono- to a tetraphosphate nucleotide (Fig. 1). A similar phosphate chain-length dependency was observed with thymine, guanine, uracil and cytosine nucleotides (data not shown). The specificity of protection is further demonstrated by the differential potencies among the common purine and pyridimine triphosphate nucleotides (Fig. 2) Although an intact nucleotide is required (Fig. 3), phosphate hydrolysis is apparently not involved (Table I).

Nucleotide-mediated protection from diphtheria toxin undoubtedly results from an antagonism of toxin-receptor binding. The molecular event responsible for the antagonism is not entirely clear, although some possibilities can be ruled out. One explanation is that nucleotides act by chelating a cation required for toxin-receptor binding. This is quite unlikely because of the considerable differences in inhibitory potencies of the common triphosphate nucleotides (Fig. 2), while their affinities for cations are essentially identical (21). Another possibility is that nucleotides bind to the toxin, not the receptor, and somehow prevent toxin-receptor interaction. This explanation is also unlikely since we measured the binding of

radiolabeled adenosine tri- , di-, and monophosphates to diphtheria toxin found only a low level, nonspecific association (data not shown). Furthermore the monophosphate bound to diphtheria toxin as well or better than adenosine triphosphate, a situation quite unlike the protective effect. Thus, we are led to the conclusion that nucleotides antagonize diphtheria toxin-receptor binding by occupying the toxin binding site on the receptor and sterically hindering the interaction, or by binding to an allosteric control site which converts the receptor to a form incapable of interacting with diphtheria toxin. Whichever the case, nucleotides clearly offer a valuable tool for future diphtheria toxin-receptor studies.

It is by no means unprecedented to find that exogenous nucleotides affect the action of a toxin or hormone. Rodbell and his associates described an obligatory role of guanyl nucleotides in glucagon activation of adenylate cyclase (5), concluding that guanyl and, to a much lesser degree, other nucleotides regulated glucagon binding by an allosteric action (11, 18). Cassel and Selinger presented evidence that inhibition of guanosine triphosphate hydrolysis may be the mechanism by which cholera toxin activates adenylate cyclase (2). Since both these examples of nucleotide regulation involved the adenylate cyclase system, we considered the possibility that our data also reflected some relationship between this enzyme and diphtheria toxin action. Experiments to date, however, have given uniformly negative results.

Nucleotides have also been known to affect a hormone not involved with adenylate cyclase. Chang and Cuatrecasas (3) and Loren et al. (12) found that exogenous adenosine triphosphate inhibited insulin-stimulated

glucose transport. It was agreed that the effect probably resulted from phosphorylation of a membrane component involved in transmission of the signal from the insulin receptor to the carrier system. Since we can measure a direct nucleotide effect on diphtheria toxin binding, and since the nonhydrolyzable nucleotide analog found to be ineffective by Chang and Cuatrecasas is fully potent in our system, it is unlikely that phosphorylation mediates the nucleotide effect seen here. Nevertheless we find it curious that nucleotides can affect toxins or hormones having widely diverse mechanisms of action and suggest that the effects of nucleotides on other hormone or toxin systems should be investigated.

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TABLE I

Effect of Nonhydrolyzable ATP Analogs on Cytotoxicity and Toxin-Cell binding

Agent	% Cell survival*	% Inhibition of toxin-cell binding‡
Adenosine-5'-triphosphate	81	84
β-γ-methyladenosine-5'-triphosphate	74	81
β-γ-adenylyl-imidophosphate	88	82
Control	19	0

*Cells were challenged with 0.05 ng/ml toxin for 3 h at 37°C in the presence or absence (control) of 0.8 mM indicated agent. Survival was measured as in Materials and Methods.

‡Cells were incubated at 4°C with 0.03 g/ml ^{125}I-toxin or ^{125}I-toxin plus 3 μg/ml unlabeled toxin for 12 h in the presence or absence of 1 mM agent. Specific binding was measured as in Materials and Methods.

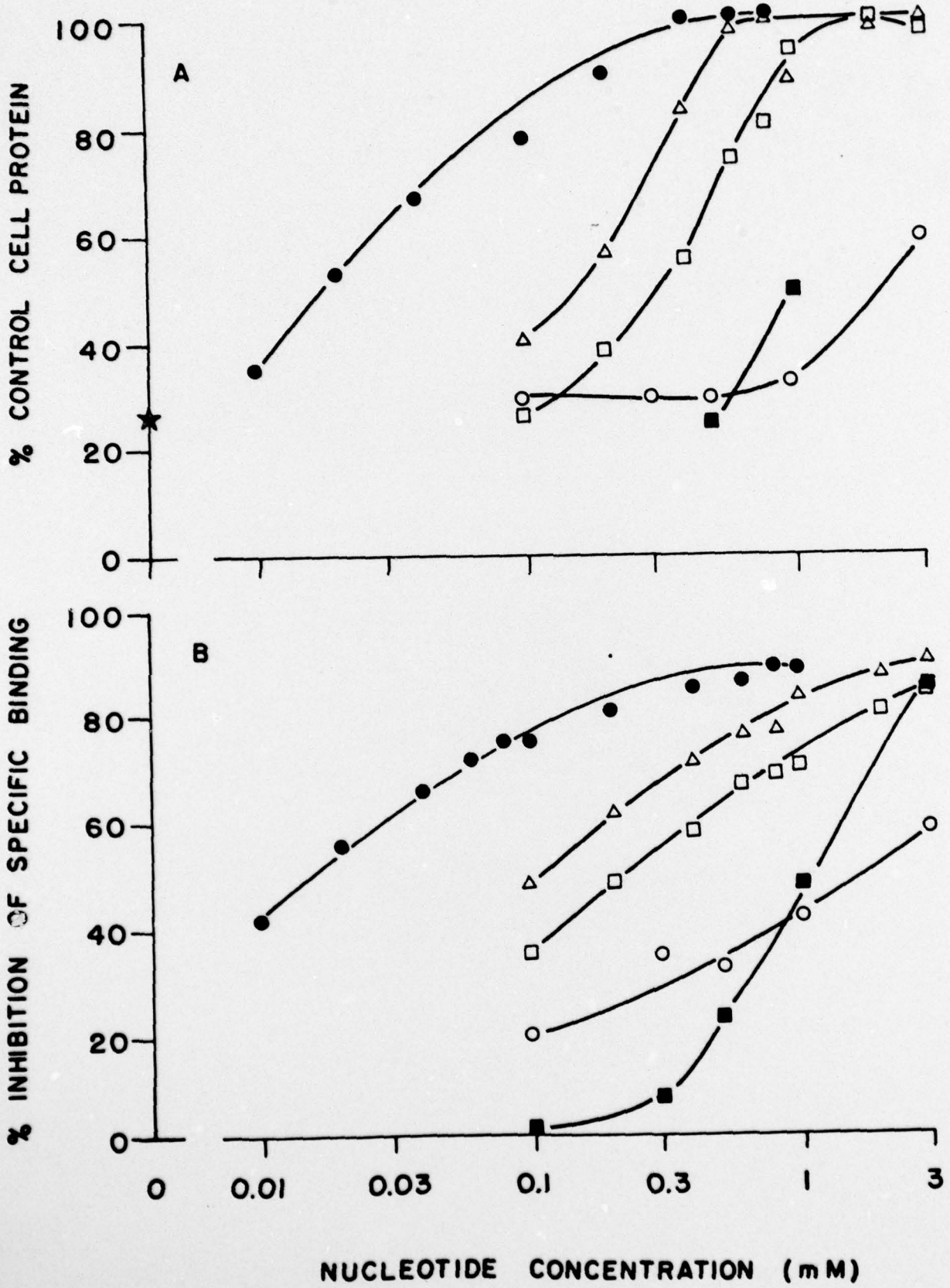
Figure Legends

FIGURE 1. Effects of adenine nucleotides on diphtheria toxin-induced cytotoxicity and ^{125}I-diphtheria toxin-cell binding. A. Effects on cytotoxicity. Vero cells were challenged with 0.05 ng/ml toxin in the presence of various nucleotides as explained in Materials and Methods. (●) adenosine-5'-tetraphosphate; (△) adenosine-5'-triphosphate; (□) adenosine-5'-diphosphate; (○) adenosine-5'-monophosphate; (■) tripolyphosphate; (star) toxin only. B. Effects on binding. ^{125}I-diphtheria toxin (0.03 μg/ml) with or without 3 μg/ml unlabeled toxin was incubated with Vero cells in the presence of indicated nucleotides at 4°C for 12 h. Specific binding was then determined as described in Materials and Methods. Symbols same as part A. Control bound cpm = 27,000.

FIGURE 2. Effects of common triphosphate nucleotides on diphtheria toxin-induced cytotoxicity and ^{125}I-diphtheria toxin-cell binding. A. Effects on cytotoxicity. Vero cells were challenged with 0.1 ng/ml toxin in the presence of various nucleotides as explained in Materials and Methods. (○) thymidine-5'-triphosphate; (□) adenosine-5'-triphosphate; (△) uridine-5'-triphosphate; (●) guanosine-5'-triphosphate; (■) cytidine-5'-triphosphate; (▲) tripolyphosphate; (star) toxin only. B. Effects on binding. ^{125}I-diphtheria toxin (0.03 μg/ml) with or without 3 μg/ml unlabeled toxin was incubated with Vero cells in the presence of the indicated nucleotides at 4°C for 12 h. Specific binding was then determined as described in Materials and Methods. Symbols same as part A. Control bound cpm = 9,000.

FIGURE 3. Effects of nucleotide components on diphtheria toxin-induced cytotoxicity and ^{125}I-diphtheria toxin-cell binding. A. Effects on cytotoxicity. Vero cells were challenged with 0.1 ng/ml toxin in the presence of 0.1 mM of the indicated agent and cytotoxicity determined as described in Materials and Methods. Error bars indicate standard error of the mean. (T) thymidine; (PPP) tripolyphosphate; (TPP) thymidine-5'-triphosphate. B. Effects on binding. ^{125}I-diphtheria toxin (0.03 µg/ml) with or without 3 µg/ml unlabeled toxin was incubated with Vero cells in the presence of the indicated agent at 4°C for 12 h. Specific binding was then determined as described in Materials and Methods. Symbols same as part A. Error bars indicate standard error of the mean. Control bound cpm = 17,000.

FIGURE 4. Double-reciprocal plot of adenosine triphosphate effects on ^{125}I-diphtheria toxin-cell binding. Vero cells were seeded in microtiter plates and on the day of the experiment (18,000 cells/well) the medium was changed to 0.1 ml of Hanks' 199 with 10% fetal calf serum and 25 mM Hepes, pH 7.4. Specific binding of ^{125}I-diphtheria toxin was measured in the presence of the indicated concentration of adenosine-5'-triphosphate in the usual manner (4°C, 12 h). Lines were fitted by regression analysis. (O) control; (□) 0.5 mM adenosine triphosphate; (Δ) 1.0 mM adenosine triphosphate.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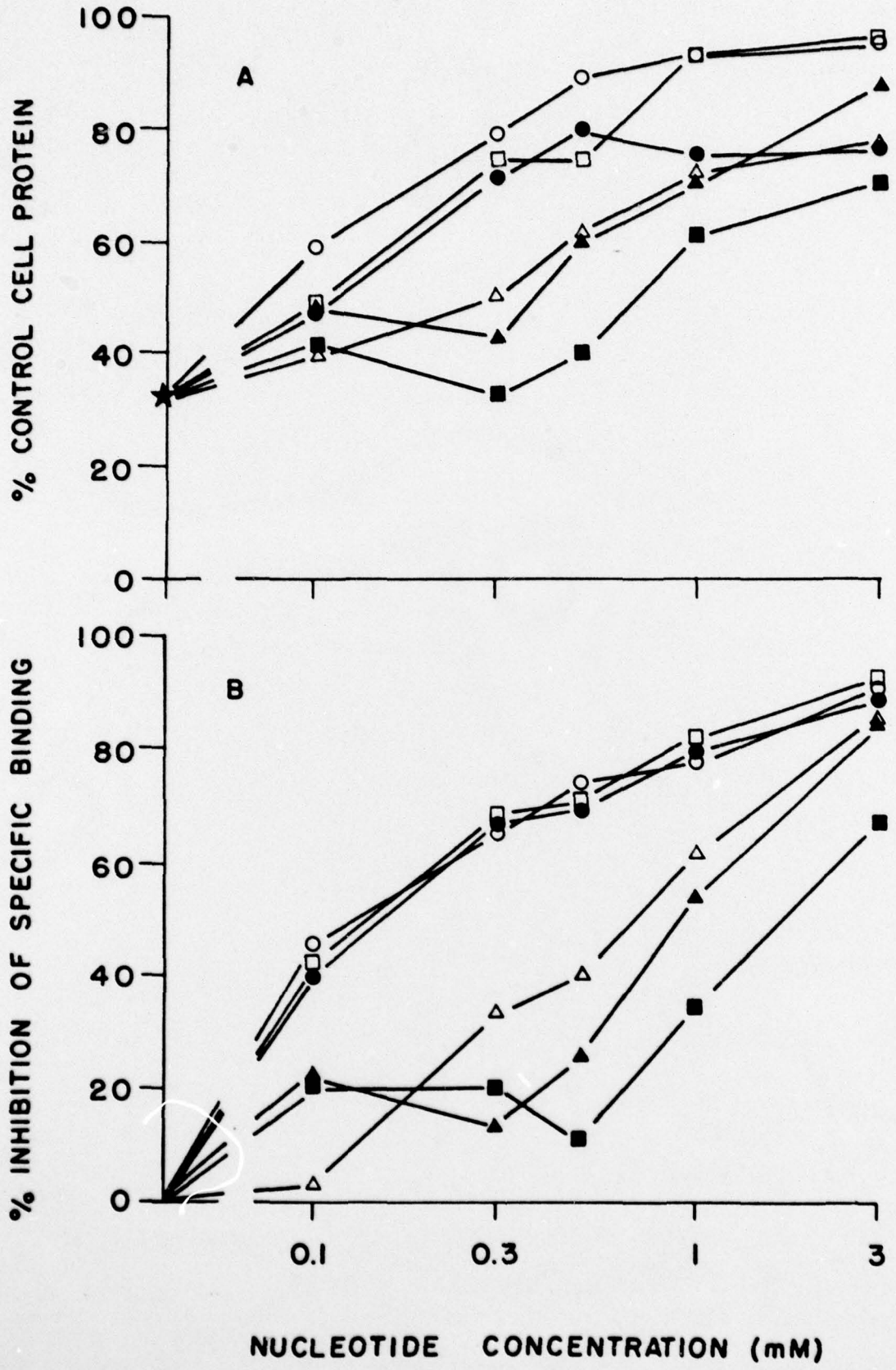
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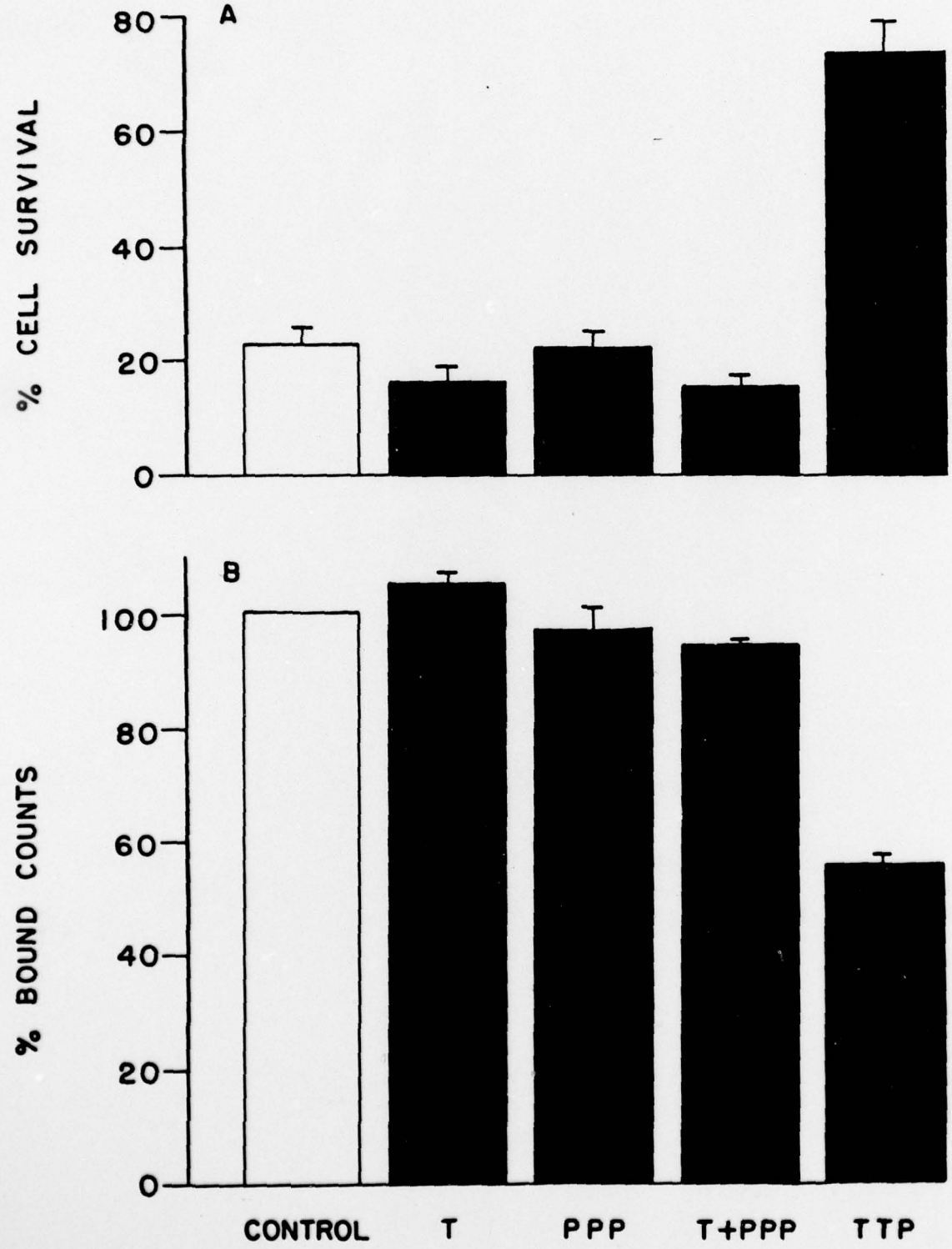
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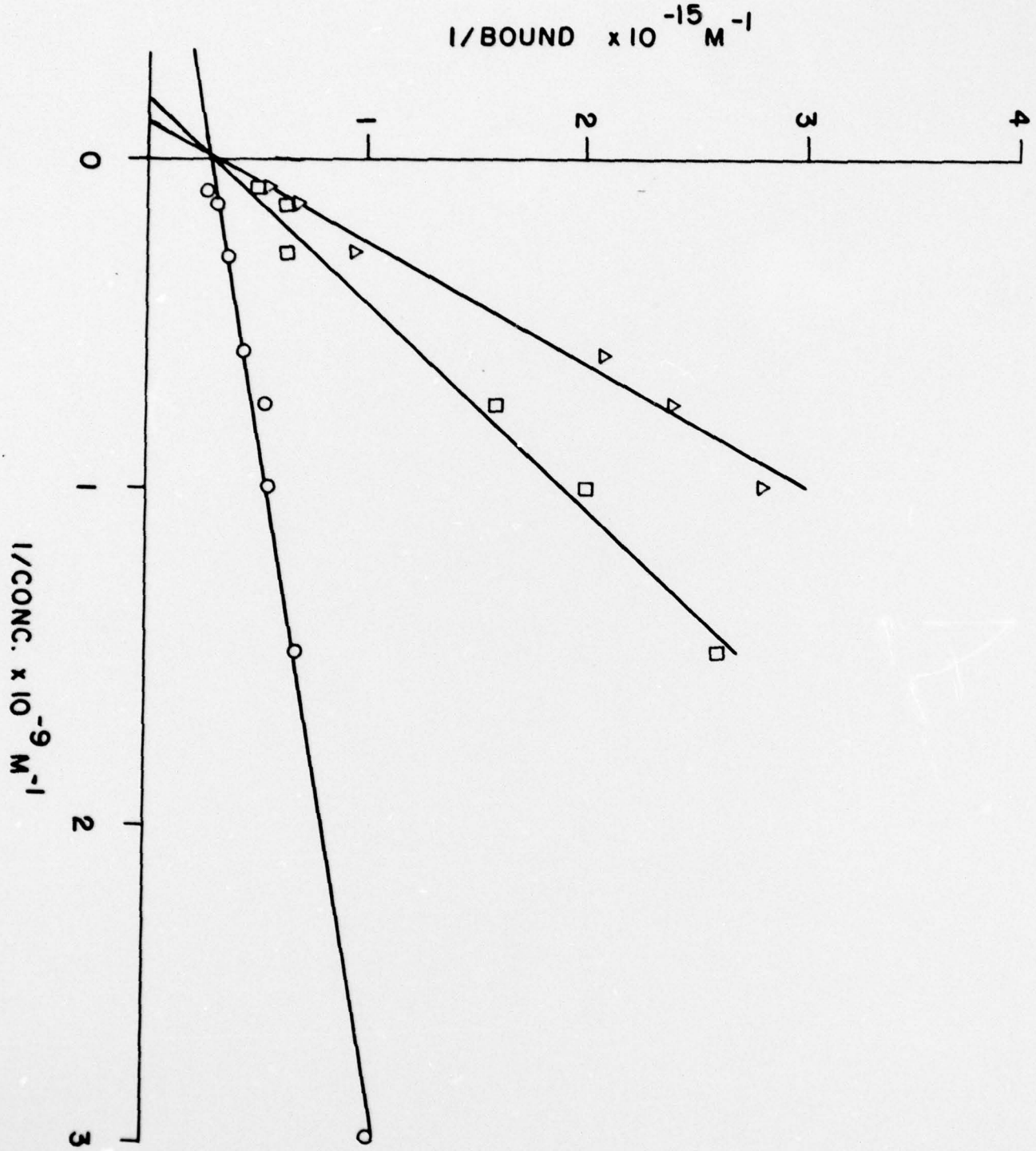
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